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Purification and Some Biological Properties of Asparaginase from *Azotobacter vinelandii*

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Asparaginase was found in the soluble fraction of cells of *Azotobacter vinelandii*, and its activity remained the same during growth of the organism in a nitrogen-free medium. The specific activity and the yield of *A. vinelandii* increased twofold in the presence of ammonium sulfate. Within limits, the temperature (30 to 37°C) and pH (6.5 to 8.0) of the medium showed little effect on the levels of enzyme activity. The enzyme was purified to near homogeneity by standard methods of enzyme purification, including affinity chromatography, and had optimum activity at pH 8.6 and 48°C. The approximate molecular weight was 84,000. The apparent K_m value for the substrate was 1.1×10^{-4} M. Metal ions or sulfhydryl reagents were not required for enzyme activity. Cu^{2+}, Zn^{2+}, and Hg^{2+} showed concentration-dependent inhibition, whereas amino and keto acids had no effect on the enzyme activity. Asparaginase was stable when incubated with rat serum and ascites fluid. The enzyme had no effect on the membrane of sheep erythrocytes and did not inhibit the incorporation of radioactive precursors into deoxyribonucleic acid, ribonucleic acid, and protein in Yoshida ascites sarcoma cells. Asparaginase activity was not detected in the tumor cells.

Asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) isolated from several biological sources has been shown to possess antitumor activity (7, 25). The enzyme from *Azotobacter vinelandii* possesses antitumor activity and also interesting inducible properties (11). In this paper, we present further studies on the regulation of synthesis, purification, and properties of the enzyme.

MATERIALS AND METHODS

Materials. L-Asparagine, D-asparagine, rifampin, chloramphenicol, diethylaminoethyl (DEAE)-cellulose, Sepharose-6B, tris(hydroxymethyl)aminomethane (Tris) and protamine sulfate were obtained from Sigma Chemical Co., St. Louis, Mo. Putrescine was purchased from Koch Light Laboratories, Colnbrook, Bucks, England. Sephadex gels (G-25, G-150, and G-200) were from Pharmacia Fine Chemicals, Uppsala, Sweden. ^{14}C-labeled *Chlorella* protein hydrolysate, [^{3}H]thymidine, and [^{3}H]uridine were obtained from Bhabha Atomic Research Centre, Bombay, India. All other chemicals were of analytical grade.

Organism and conditions of cultivation. *A. vinelandii* OP (obtained from R. H. Burris, University of Wisconsin) was maintained on nitrogen-free medium with sucrose as the carbon source (11). The growth medium contained (per liter): KH_2PO_4, 0.2 g; K_2HPO_4, 0.8 g; sucrose, 20 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$ 0.09 g; $FeCl_3 \cdot 6H_2O$, 5 mg; and $NaMoO_4 \cdot 2H_2O$, 3 mg. Whenever ammonium sulfate (1.4% wt/vol) was used in the growth medium, the phosphate buffer concentration was increased sevenfold to control pH. *A. vinelandii* cells were generally grown in 200 ml of the medium in 500-ml Erlenmeyer flasks at 30°C on a rotary shaker (250 rpm). Growth curve of the organism and asparaginase activity were determined by using an Emenvee model 1-F-10 fermentor equipped with a 12-liter vessel containing 8 liters of the medium (1 liter/min, 60 rpm, and 30°C). The cells were routinely harvested by centrifugation at 5,000 × *g* for 5 min at 0 to 4°C in an RC-2B Sorvall centrifuge and washed thrice with chilled buffered physiological saline.

Turbidity was measured in a Spectronic 20 colorimeter at 630 nm. Unless otherwise stated, all operations were carried out at 0 to 4°C. Potassium phosphate buffer (0.01 M), pH 7.4, was used throughout the experiments. In general, all experiments were done in duplicate.

Preparation of gels and ion exchangers for chromatography. The procedure for the preparation of gel for affinity chromatography was essentially as described by Kristiansen et al. (18). Chlorosuccinamic acid was prepared from D-asparagine according to the method of Holmberg (14). The melting point of these crystals was 126 to 127°C and the yield was 40%. Distilled putrescine (boiling point, 154°C; specific gravity, 0.88) was used as the spacer molecule. Cyanogen bromide was prepared as described by Hartman and Dreger (13). Six grams of cyanogen bromide was used for the activation of 100 ml of Sepharose-6B and carried out for 5 min at pH 10.5 to

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11.0. Putrescine-linked chlorosuccinamic acid was coupled to the activated Sepharose, and the absorbent was cycled several times between pH 4.0 (0.1 M acetate buffer) and 8.5 (0.1 M borate buffer) before use in a column.

Polyacrylamide gel electrophoresis was performed in 7.5% acrylamide gels at pH 8.4 according to the method of Davis (8).

The molecular weight of asparaginase was determined by gel chromatography using Sephadex G-200 in a column (2 by 57 cm; void volume, 48 ml) as described by Andrews (1).

Preparation of rat serum, ascites fluid, and cell-free extract of YAS cells. Blood from rats was collected by cardiac puncture and allowed to clot for 2 h at room temperature (28°C) and 8 to 10 h at 0 to 4°C. The blood was centrifuged at 10,000 × *g* for 10 min, and the serum was collected.

The Yoshida ascites sarcoma (YAS) cells were aspirated from the peritoneal cavity of the tumor-bearing rats on day 4 and centrifuged at 500 × *g* for 5 min to pellet out YAS cells. The supernatant was centrifuged at 10,000 × *g* for 10 min to clear the ascites fluid. This ascites fluid was used for incubation with the enzyme. The packed YAS cell pellet was washed by resuspending in saline (four times, 50 ml each time), and the contaminating erythrocytes (RBC) were removed by lysing the cells in cold distilled water for 2 min. This was followed by centrifugation at 10,000 × *g* for 10 min to get highly packed cell pellets. Five grams (wet weight) of washed YAS cells was suspended in 30 ml of 0.02 M Tris-hydrochloride buffer, pH 7.4, and homogenized for 10 min in a tissue homogenizer. The extract obtained was centrifuged at 12,000 × *g* for 10 min, and the supernatant obtained was used for assaying asparaginase activity.

Effect of asparaginase on mammalian RBC membrane. The hemolytic activity of asparaginase was tested by its effect on sheep RBC. A washed suspension of sheep RBC (3×10^7 cells/ml) in buffered 0.15 M NaCl, pH 7.4, was incubated with 50, 100, 150, and 200 U of the enzyme for 30 min at 37°C. The incubation mixture was centrifuged, and the supernatant was used to measure the hemoglobin released at 540 nm in a spectrophotometer (16). Loss of hemoglobin was expressed as percentage of total cellular content in the untreated cells.

Effect of asparaginase on deoxyribonucleic acid, ribonucleic acid, and protein synthesis in isolated tumor cells. The YAS cells (1.4×10^7 cells/ml) were suspended in Eagle minimal essential medium and incubated with 50 to 200 enzyme units at 37°C for 10 min. This was followed by the addition of either [^{3}H]thymidine, [^{3}H]uridine, or ^{14}C-labeled *Chlorella* protein hydrolysate (each at 1 μCi/ml) and incubated further at 37°C for 30 min. The tubes were then chilled, and 0.1 ml of 1% Triton X-100 was added to tubes containing YAS cells. Aliquots of the above samples were spotted onto filter papers, processed, and counted in a Beckman LS-100 scintillation counter (5).

Purification of asparaginase. Three methods were used to extract asparaginase of *A. vinelandii*. The butanol extraction method used by Shethna et al. (29) for the extraction of iron-sulfur proteins did yield an extract of higher specific activity (6.5), but the viscous extract was unsuitable for further purification of the enzyme.

The preparation of acetone powder of *A. vinelandii* and extraction of asparaginase into buffer resulted in extracts of low specific activity (2.0) and yielded multiple enzyme peaks during DEAE-cellulose column chromatography.

The final procedure adopted for enzyme extraction and purification was as follows. Crude cell-free extracts of asparaginase were obtained by grinding *A. vinelandii* cells (20 g, wet weight) along with glass powder (40 g), using 200 ml of 0.01 M phosphate buffer for extraction. The cell debris was removed by centrifugation at 27,000 × *g* for 20 min. The crude extract was treated with protamine sulfate (1 mg per 10 mg of protein) to precipitate the nucleic acids, which were removed by centrifugation at 27,000 × *g* for 10 min. The supernatant was subjected to ammonium sulfate fractionation at pH 8.4. The protein precipitate obtained between 30 to 60% ammonium sulfate saturation, which contained most of the enzyme activity, was dissolved in a minimal amount of 0.05 M Tris-hydrochloride buffer, pH 8.4, and chromatographed on a Sephadex G-150 column (2.5 by 75 cm; void volume, 135 ml) with the same buffer containing 0.1 M KCl as the eluent. Fractions of 2.5 ml were collected at a flow rate of 15 ml/h. Most of the proteins were eluted as a major peak immediately after the void volume. The enzyme activity was associated with the minor protein peak eluted adjacent to the major protein peak. Fractions containing maximum enzyme activity (fractions 78 to 94) were pooled; then the protein was precipitated with ammonium sulfate (75% saturation) and desalted on a Sephadex G-25 column. The enzyme solution was applied to a DEAE-cellulose column (1.5 by 16 cm; flow rate, 15 ml/h) previously equilibrated with phosphate buffer of 0.02 M, pH 7.3. After being washed with 100 ml of the buffer, the column was developed with a linear gradient of 0.0 to 0.5 M KCl (75 ml each) in the same buffer. The protein was eluted as a broad peak, and the enzyme activity (eluted with 0.2 M KCl) corresponded to the major protein peak. The protein from the active fractions was precipitated with ammonium sulfate, centrifuged, dissolved in 0.1 M acetate buffer, pH 7.0, and passed through a Sephadex G-25 column. It was then applied to an affinity column (1.5 by 12 cm; flow rate, 5 ml/h) and eluted by a linear gradient of 0.0 to 1.0 M KCl (50 ml each) in the acetate buffer. The activity corresponded to the protein peak at 0.4 M KCl. The enzyme fractions were pooled and concentrated. This preparation, which showed two very closely moving bands on polyacrylamide gel electrophoresis (Fig. 1), was used for further characterization of the enzyme. The enzyme purification steps are given in Table 1.

Protein estimation and assay of asparaginase activity. Protein was determined by either the biuret method (12) or the method of Lowry et al. (21).

Asparaginase was assayed by estimating the product ammonia by nesslerization. The estimation of ammonia was carried out essentially according to the procedure described by Mortenson (22) for the assay of nitogrenase and Jayaram et al. (15) for the

assay of asparaginase. Suitable controls were included in all experiments.

The specific activity of the enzyme is expressed as units per milligram of protein. One unit is defined as the amount of protein required to liberate 1 μmol of ammonia at 37°C in 30 min.

RESULTS

Cellular distribution of asparaginase. When the particulate and supernatant fractions obtained by centrifugation of a cell-free extract of *A. vinelandii* at 100,000 × *g* were assayed for asparaginase, 80% of the activity was observed in the supernatant fraction, suggesting that it is a soluble enzyme.

Time course of cell growth and enzyme activity. The growth curve of the organism in a nitrogen-free medium and the specific activity of asparaginase in different phases of growth are given in Fig. 2. The enzyme activity remained unchanged except for a minor increase toward the end of the logarithmic phase.

Experiments done to study the effect of varying the concentration of iron ($FeCl_3 \cdot 6H_2O$, 1 to 10 mg/liter), molybdenum ($NaMoO_4 \cdot 2H_2O$, 1 to 6 mg/liter), and phosphate (1 to 10 g/liter) in the medium on asparaginase activity in crude extracts showed that none of these medium constituents had any effect on enzyme activity.

Rate of utilization of ammonium nitrogen and influence on growth of the organism. The growth pattern of the organism in a fermentor in growth medium and the growth medium containing ammonium sulfate is given in Fig. 3. The growth rate was high with added ammonium sulfate, and the yield of the organism was

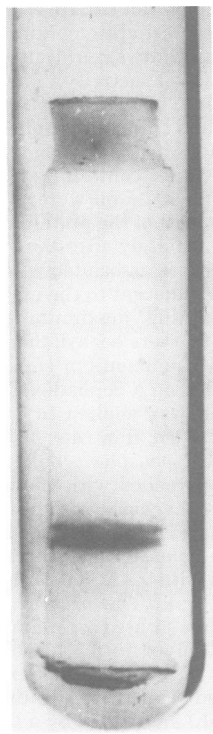

FIG. 1. *Polyacrylamide gel electrophoresis of the enzyme preparation. Protein (100 μg) was loaded, and the protein was stained with Coomassie blue.*

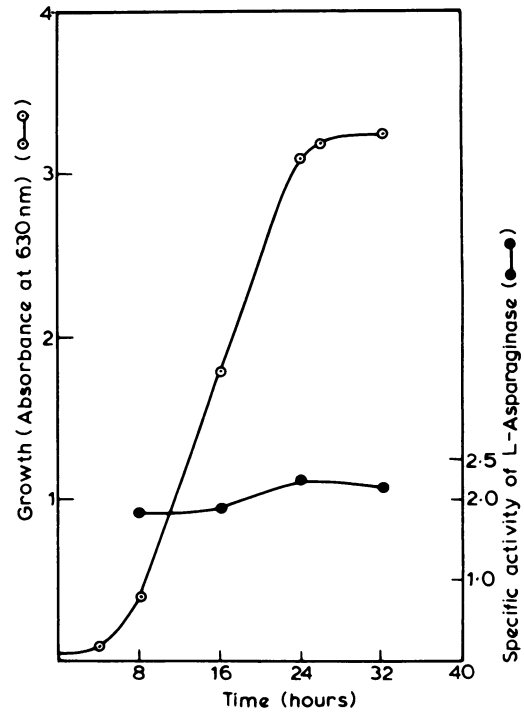

FIG. 2. *Time course of growth and enzyme activity in nitrogen-free medium.*

TABLE 1. *Purification of asparaginase from A. vinelandii*

Purification step	Total protein (mg)	Total activity (U)	Sp act	Fold purification	Yield (%)
Crude extract	5,400	21,600	4.0		100
Protamine sulfate tratment	4,332	19,490	4.5		90
$(NH_4)_2SO_4$ fractionation	2,100	13,650	6.5	1.6	63
Sephadex G-150	242	4,356	18	4.5	21
DEAE-cellulose	21	1,050	50	12.5	5
Affinity chromatography	8.4	622	74	18.5	2.8

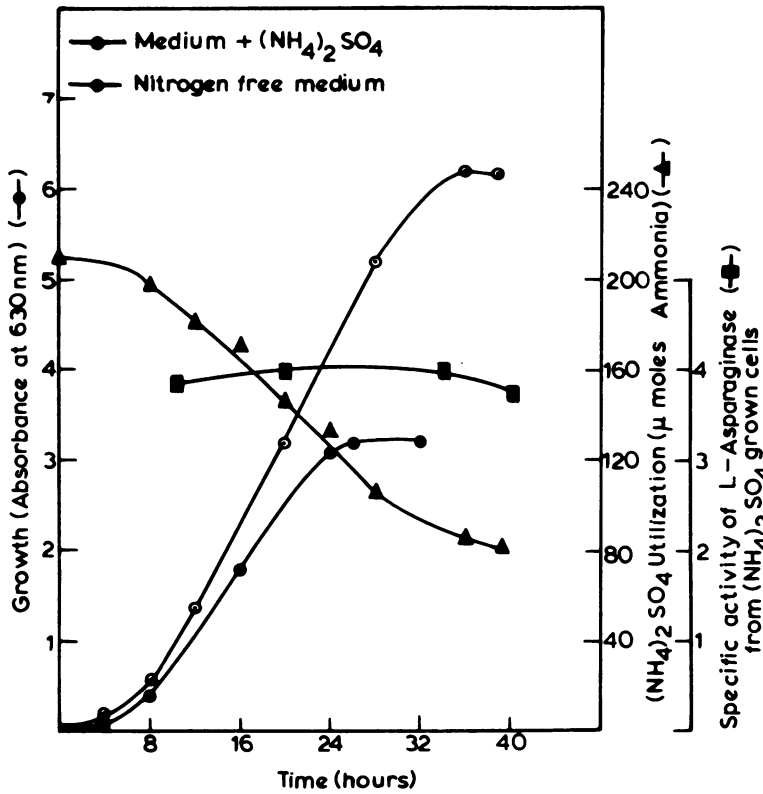

FIG. 3. *Rate of utilization of ammonium nitrogen and influence on growth of the organism.*

doubled. The specific activity of the enzyme remained at the "induced" level irrespective of the amount of ammonium nitrogen present in the medium during growth. Ammonium utilization continued, but at a low rate even in the stationary phase of growth. The specific activity of the enzyme remained at the constitutive level.

Demonstration of de novo synthesis of asparaginase in response to the inducer. Figure 4 shows the linear rate of incorporation of ^{14}C-labeled amino acids into the proteins up to 20 min. Chloramphenicol and rifampin inhibited the protein synthesis to 82% in 10 min and 45% in 30 min, respectively. *A. vinelandii* cells grown in growth medium when incubated with chloramphenicol or rifampin did not show increased asparaginase activity when ammonium sulfate was added. Maximum enzyme activity was observed in 30 min after the addition of ammonium sulfate (Fig. 5).

When the ammonium sulfate was removed (by centrifugation and washing with fresh medium) and the induced cells were resuspended in fresh nitrogen-free medium, the specific activity of the enzyme was decreased by 50% within 60 min, whereas the decrease in the activity of the induced enzyme was gradual to the original level for the duration of the experiment (Fig. 6).

The relationship between the time of addition of ammonium sulfate (1.4%, wt/vol) and asparaginase formation during the growth of *A. vinelandii* indicated no significant difference in induction of enzyme synthesis (specific activity, 4.1) in all the growth phases of the organism.

Physical factors affecting the activity of asparaginase in *A. vinelandii*. Varying the pH of the growth medium and of growth medium with ammonium sulfate from 6.5 to 8.0 did not affect the enzyme activity in crude extracts, but the yield of the organism at 37°C was 20 to 25% lower than that obtained at 30°C in both growth media. The organism did not grow when incubated under stationary conditions.

General properties of asparaginase. The purified enzyme preparation showed no loss of activity when stored at −20°C for 20 days. It was stable during lyophilization and concentration with aquacide, but inactivation was observed after dialysis.

The enzyme showed optimum activity at pH 8.6 and 48°C, was stable up to 50°C, and was

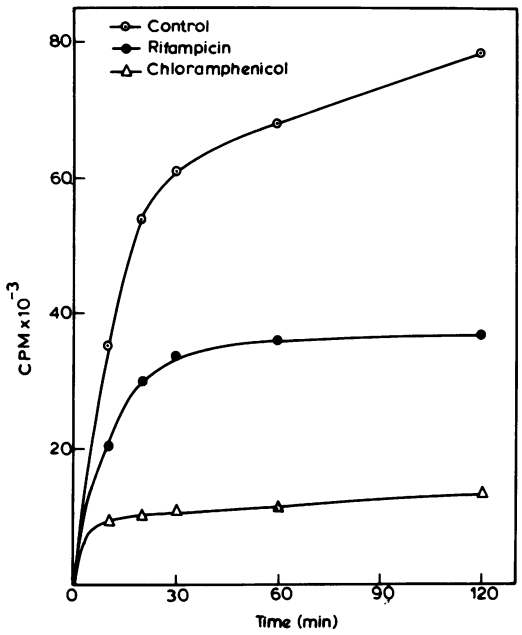

FIG. 4. *Rate of incorporation of ^{14}C-labeled amino acids into proteins of A. vinelandii. Ten milliliters of A. vinelandii culture in the logarithmic phase of growth (optical density, 2.0) was transferred into each of the three 50-ml conical flasks. ^{14}C-labeled Chlorella protein hydrolysate (specific activity, 39.3 mCi/mmol), chloramphenicol, or rifampin was added to each flask to a final concentration of 0.08 μCi/ml, 100 μg/ml, or 20 μg/ml, respectively. The two flasks with the inhibitor and a control flask without the inhibitor were incubated on the rotary shaker at 30°C. Samples of 1.5 ml were withdrawn at 10-, 20-, 30-, 60-, 90-, and 120-min intervals and added to an equal volume of 10% cold trichloroacetic acid. Further processing of these cell samples for counting radioactivity was done according to the method of Shaila et al. (28).*

inactivated at 65°C. The apparent K_m value calculated from the Lineweaver-Burk plot was 1.1×10^{-4} M.

The approximate molecular weight as calculated from the calibration curve was 84,000.

Effect of metal ions, metal-chelating agents, and some other common enzyme inhibitors (Table 2). L-Aspartic acid, L-glutamic acid, L-histidine, L-methionine, L-leucine, L-isoleucine, and L-threonine, as well as pyruvate, citrate, malate, succinate, and α-ketoglutarate, had no effect on the enzyme activity, nor did 2-mercaptoethanol, dithioerythreitol, and reduced glutathione.

Effect of rat serum and ascites fluid on the asparaginase of *A. vinelandii*. Incubation of asparaginase with rat serum or ascites fluid for 2 h at 37°C resulted in a decrease by 10 to 15% of its activity, showing that the enzyme is relatively stable to these physiological fluids. The asparaginase caused no lysis of sheep RBC at all levels tested.

Similarly, the enzyme had no effect on either the uptake of radioactive precursors of the macromolecules or the synthesis of deoxyribonucleic acid, ribonucleic acid, and protein of cultured YAS cells.

The cell-free extract of normal YAS cells had no detectable asparaginase activity.

DISCUSSION

Several microorganisms have been reported (2) to produce extracellular asparaginase, but this is not the case in *A. vinelandii*. All endocellular asparaginases reported so far, including the present one, were found in the soluble fraction of the extract. In *A. vinelandii* the

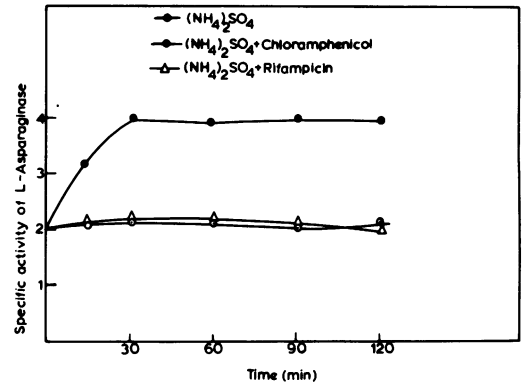

FIG. 5. *Demonstration of de novo synthesis of asparaginase in the presence of inducer of the enzyme. This experiment was done by studying the increase in the enzyme activity in presence and absence of chloramphenicol or rifampin.*

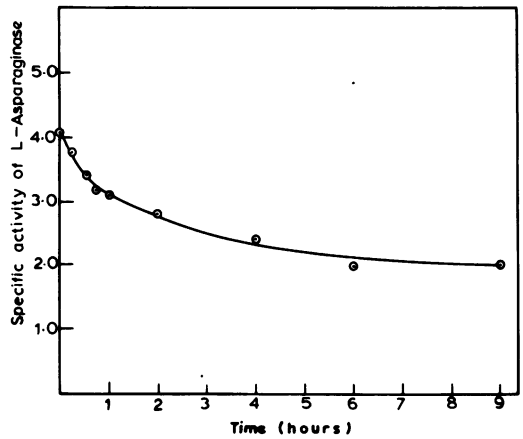

FIG. 6. *Changes in the specific activity of asparaginase after the removal of inducer from the medium.*

TABLE 2. *Effect on asparaginase of metal ions, metal-chelating agents, and other inhibitors*

Addition	% Inhibition at concn of:				
	1 mM	2 mM	3 mM	10 mM	20 mM
Mg^{2+}	100		98		
Hg^{2+}	32		24		
Fe^{2+}	100		24		
Ca^{2+}	100		98		
Co^{2+}	100		82		
Cu^{2+}	35		35		
Mn^{2+}	98		100		
Zn^{2+}	50		44		
Ethylenediaminetetraacetic acid				100	100
α,α-Dipyridyl				100	100
Control				100	100
None		100			
Hydroxylamine	95	65			
Hydrazine	75	70			
Sodium azide	95	90			
Potassium cyanide	90	40			
Potassium ferricyanide	75	65			
Atabrin	75	65			
pHMB	0.0	0.0			
Iodoacetate	87	58			
N-ethymaleimide	0.0	0.0			

enzyme was found to be constitutive. There is no definite relationship between the growth phase and increase in the specific activity of asparaginase.

In *Erwinia aroideae*, Beyaert and Voets (4) have reported that buffering the medium decreased the activity of asparaginase, but in *A. vinelandii*, an increased concentration of potassium phosphate buffer had no effect on enzyme activity. Suboptimal amounts of iron in the medium resulted in the appearance of a greenish-yellow fluorescent peptide whose concentration increased with time of incubation (6). Iron and molybdenum are not essential for the production of asparaginase by *A. vinelandii*.

In *A. vinelandii*, although asparaginase is constitutive, its activity is increased by ammonium nitrogen. De novo synthesis of this enzyme protein by the addition of inducers (11) is evident from the inhibition of such an increase by chloramphenicol or rifampin. A similar observation has been made in *Pseudomonas* (10). It is possible that the enhanced activity is due to the formation of an isoenzyme. However, the enzyme preparations obtained from the induced cells did not show different pH optima. A two- to threefold increase in specific activity of asparaginase in crude cell-free extracts has been noted in *Escherichia coli* B (3) and *Pseudomonas* (26), but isoenzyme synthesis has been reported in a different strain of *E. coli* (17).

The time needed for reaching maximum enzyme activity in response to ammonium sulfate addition in *A. vinelandii* is longer than in *Pseudomonas:* within 3 min of the addition of inducer, the enzyme activity increased in *Pseudomonas*, but reverted to the control level in 12 to 20 min (3). Further growth of the organism caused a continuous decrease in enzyme activity. In *Azotobacter*, the maximal enzyme activity is reached in 30 min and is maintained at the same level during further growth of the organism in the presence of ammonium sulfate. *E. aroideae* (20) is not able to grow in the presence of ammonium nitrogen. The *A. vinelandii* asparaginase is induced in all stages of growth.

Low oxygen levels in *E. coli* (23) and anaerobic conditions in *Bacterium cadaveris* (9) have been shown to increase enzyme production. In *A. vinelandii*, no growth is observed under stationary conditions.

Butanol extraction of asparaginase is a convenient procedure for handling large quantities of cells on a preparative scale. Asparaginase from *A. vinelandii* was stable during butanol treatment of cells. Our application of this procedure to *E. coli* B, *Pseudomonas, Proteus vulgaris, Serratia marcescens, Mycobacterium smegmatis,* and *Saccharomyces cerevisiae* was not successful. Acetone powder preparation of *A. vinelandii* cells did not improve the fold purification of the enzyme. Chromatography of crude cell-free extracts on an affinity column showed a high nonspecific binding of proteins. This could be due to the fact that the crude enzyme extracts from *A. vinelandii* are known to contain a high percentage of particulate enzymes, iron-sulfur proteins, nitrogen-fixing enzymes, colored pigments, etc., which could greatly interfere with other proteins during purification.

Asparaginases isolated from various biological sources differ considerably in biochemical properties and biological activity against tumors. Asparaginase from *A. vinelandii* had an optimum pH different from asparaginases of *Bacillus coagulans* (19), guinea pig serum (31), and *S. marcescens* (27), which have been reported to have broad pH optima. Sensitivity of *A. vinelandii* asparaginase to *p*-hydroxymercuribenzoate (pHMB), *N*-ethylmaleimide, iodoacetate, and heavy-metal ions (Hg^{2+}, Cu^{2+}, and Zn^{2+}) indicates that the activity of the enzyme may depend upon sulfhydryl groups. On the other hand, asparaginase from *E. coli* B (10) has been shown to be insensitive to these agents, whereas guinea pig serum asparaginase is inhibited by pHMB but not by iodoacetate or *N*-ethylmaleimide (30). The *A. vinelan-*

dii enzyme is less sensitive to iodoacetate than to pHMB and *N*-ethylmaleimide (Table 2).

Asparaginases isolated from guinea pig serum (31) and *Erwinia* (24) have a molecular weight close to 130,000, whereas asparaginase of *A. vinelandii* resembles that of *B. coagulans* (19).

The specific activity of the enzyme is not high in purified preparations but is active against YAS cells in rats (11).

LITERATURE CITED

1. **Andrews, P.** 1964. Estimation of the molecular weights of proteins by Sephadex gel-filtration. Biochem. J. 91:222-233.
2. **Arima, K., T. Sakamoto, C. Araki, and G. Tomura.** 1972. Production of extracellular L-asparaginase by microorganisms. Agric. Biol. Chem. 36:356-361.
3. **Berezov, T. T., G. Z. Hisamov, and V. A. Zanin.** 1972. Regulation of asparaginase synthesis in *Pseudomonas boreopolis* 526. FEBS Lett. 28:10-12.
4. **Beyaert, G., and J. P. Voets.** 1970. Factors affecting the production of L-asparaginase by *Erwinia aroideae*. (English translation.) 35:1091-1097.
5. **Bollum, F. J.** 1968. Filter paper disk techniques for assaying radioactive macromolecules. Methods Enzymol. 12B:169-177.
6. **Bulen, A. W., and J. R. Le Compte.** 1962. Isolation and properties of a yellow-green fluorescent peptide from *Azotobacter* medium. Biochem. Biophys. Res. Commun. 9:523-528.
7. **Cooney, D. A., and R. E. Handschumacher.** 1970. L-asparaginase and L-asparagine metabolism. Annu. Rev. Pharmacol. 10:421-440.
8. **Davis, B. J.** 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. 121:404-427.
9. **Eremenko, V. V., L. P. Evseev, and A. Y. Nikolaev.** 1968. Asparaginase of *Bacterium cadaveris*. Mikrobiologiya 37:207-212 (in Russian. English translation in Microbiology [USSR] 37:173-177.)
10. **Evseev, L. P., A. Y. Nikolaev, V. V. Eremenko, and S. R. Mardashev.** 1967. Induced synthesis of asparaginase and glutaminase by *Pseudomonas*. Biokhimiya 32:873-875 (in Russian).
11. **Gaffar, S. A., and Y. I. Shethna.** 1975. Partial purification and antitumour activity of L-asparaginase from *Azotobacter vinelandii*. Curr. Sci. 44:77-79.
12. **Gornall, A. G., C. S. Bradawill, and M. M. David.** 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177:751-766.
13. **Hartman, W. W., and E. E. Dreger.** 1950. Cyanogen bromide preparation. Org. Syn. 2:150.
14. **Holmberg, B.** 1926. Stereochemische Studien. XIII. Über α-Chlor-succinamidsäuren. Chem. Ber. 59:1569-1572.
15. **Jayaram, H. N., T. Ramakrishnan, and C. S. Vaidyanathan.** 1968. L-asparaginases from *Mycobacterium tuberculosis* strains $H_{37}R_v$ and $H_{37}R_a$. Arch. Biochem. Biophys. 126:165-174.
16. **Kabat, E. A., and M. M. Mayer.** 1967. Experimental immunity. Charles C Thomas, Publisher, Springfield, Ill.
17. **Khernadi, F., K. Polya, and A. Darochi.** 1972. Selection of *Escherichia coli* strains producing large amounts of L-asparaginase (English translation). Microbiology (USSR) 41:24-28.
18. **Kristiansen, T., M. Einarsson, L. Sundberg, and J. Porath.** 1970. Purification of L-asparaginase from *Escherichia coli* by specific adsorption and desorption. FEBS Lett. 7:294-296.
19. **Law, A. S., and J. C. Wriston, Jr.** 1971. Purification and properties of *Bacillus coagulans* L-asparaginase. Arch. Biochem. Biophys. 147:744-752.
20. **Liu, F. S., and J. E. Zajic.** 1972. L-Asparaginase synthesis by *Erwinia aroidiae*. Appl. Microbiol. 23:667-668.
21. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
22. **Mortenson, L. E.** 1961. A simple method for measuring nitrogen fixation by cell-free enzyme preparations of *Clostridium pasteurianum*. Anal. Biochem. 2:216-220.
23. **Netrval, J.** 1973. Effect of organic and amino acids on L-asparaginase production by *Escherichia coli*. Arch. Mikrobiol. 92:345-352.
24. **North, A. C. T., H. E. Wade, and K. A. Cammack.** 1969. Physicochemical studies of L-asparaginase from *Erwinia carotovora*. Nature (London) 224:594-595.
25. **Oettgen, H. F., L. Tallal, C. C. Tan, M. L. Murphy, B. D. Clarkson, R. D. Golbey, I. H. Krakoff, D. A. Karnofsky, and J. H. Burchenal.** 1973. Clinical experience with L-asparaginase. Recent Results Cancer Res. 33:219-233.
26. **Robison, R. S., and B. Berk.** 1969. L-asparaginase synthesis by *Escherichia coli* B. Biotechnol. Bioeng. 11:1211-1225.
27. **Rowley, B., and J. C. Wriston, Jr.** 1967. Partial purification and antilymphoma activity of *Serratia marcescens* L-asparaginase. Biochem. Biophys. Res. Commun. 28:160-165.
28. **Shaila, M. S., K. P. Gopinathan, and T. Ramakrishnan.** 1973. Protein synthesis in *Mycobacterium tuberculosis* $H_{37}R_v$ and the effect of streptomycin in streptomycin-susceptible and -resistant strains. Antimicrob. Agents Chemother. 4:205-213.
29. **Shethna, Y. I., P. W. Wilson, and H. Beinert.** 1966. Purification of a non-heme iron protein and other electron transport components from *Azotobacter* extracts. Biochim. Biophys. Acta 113:225-234.
30. **Tower, D. W., E. L. Peters, and W. C. Curtis.** 1963. Guinea pig serum L-asparaginase. J. Biol. Chem. 238:983-993.
31. **Yellin, T. O., and J. C. Wriston, Jr.** 1966. Purification and properties of guinea pig serum asparaginase. Biochemistry 5:1605-1612.